Calcimimetics inhibit renal pathology in rodent nephronophthisis

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The development and progression of renal cysts appears to be driven by reduced cellular calcium and increased cyclic adenosine monophosphate (cAMP) from G-protein-coupled receptors. To test whether treatment with a calcimimetic that stimulates the G-protein-coupled calcium-sensing receptor might normalize cystic epithelial cell intracellular calcium and cAMP, thereby inhibiting cyst progression, we used pcy mice. These animals develop cysts principally in the collecting duct, as do humans with nephronophthisis (NPHP). We administered the calcimimetic R-568 mixed in their food at early or late stages in the pathogenesis of cyst formation. The treatment reduced cyst enlargement, and the early treatment inhibited development of renal fibrosis. Although the effect of later treatment was more modest, both stages of the disease responded positively to treatment. Additionally, R-568 decreased total kidney cAMP in the pcy mice and, *in vitro*, decreased cAMP levels and cell proliferation, while increasing intracellular calcium in immortalized human autosomal recessive polycystic kidney disease renal epithelial cells. The latter two effects were unique to R-568 and not replicated by raising extracellular calcium. Thus, treating pcy mice with R-568 was effective in reducing cyst progression in this rodent model of NPHP. Direct studies will be needed to determine whether these results can be applied to the human disease.

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Cystic kidney diseases due to genetic disorders are a leading cause of kidney failure and death in children and adults. Two childhood forms of renal cystic disease, autosomal recessive polycystic kidney disease (ARPKD) and nephronophthisis (NPHP), are characterized by collecting-duct cysts.[1,2] Fibrocystin/polyductin, the protein encoded by the *PKHD1* gene mutated in ARPKD, and nephrocystin 3, from the *NPHP3* gene, are localized to primary cilia.[3] These cystic conditions are associated with abnormalities in cilia structure or function, altered regulation of cellular cyclic adenosine monophosphate (cAMP) and intracellular calcium, and increased epithelial cell proliferation and apoptosis.[4] Thus, interventions that reverse ciliary dysfunction, alter proliferation, or reverse the alterations in intracellular calcium/cAMP may impact the progression of the disease.

Calcium is regulated by several hormones, including parathyroid hormone (PTH) and calcitriol, acting on bone, kidney, and the intestine.[5] Extracellular calcium influences intracellular actions through its binding to the CaSR, a G-protein-linked receptor.[6] Activation of the CaSR increases intracellular calcium and decreases cAMP generation,[7] effects that would oppose the abnormalities in PKD. In the collecting duct, the CaSR is located apically and appears to sense increased luminal calcium and decreases arginine vasopressin-dependent aquaporin-2 expression, perhaps to dilute the urine and prevent hypercalciuria.[8] Type I agonists are inorganic polyvalent cations (for example, calcium and magnesium) and activate the CaSR, without the need for other agonists. In contrast, type II agonists, or calcimimetics, are positive allosteric modulators of the CaSR. The first generation type II CaSR agonist, R-568, was manufactured by NPS (Oread Labs, Lawrence, KS), and the second generation of calcimimetic, Cinacalcet, was developed by Amgen (Amgen Thousand Oaks, CA) and is approved by the FDA for use in the treatment of secondary hyperparathyroidism in patients on dialysis. The primary difference between R-568 and Cinacalcet are the P450 drug interactions.[9] We have previously demonstrated that treatment with R-568 inhibited cyst growth in the Cy/+ model of cystic kidney disease. Kidney size, cyst volume density, fibrosis, and renal function were all improved between 34 and 38 weeks of age, a time when there is cyst growth of all tubular segments.[10] In the

present study, we determined the effect of R-568 on an orthologous model of NPHP, the pcy mice, with cyst formation derived principally from the collecting duct. The results demonstrate that R-568 is effective in the prevention of renal cystic disease progression in this animal model.

RESULTS

The effect of R-568 on the prevention of cystic disease in pcy mice

NPHP (pcy) mice and normal CD1 mice were treated with control or R-568 for 11 weeks, starting from 4 weeks of age. There was no significant gender dimorphism in the cystic disease progression in the pcy mouse model; therefore, male and female data were combined. The observed changes in serum biochemistries are shown in Table 1. There were significant elevations in serum blood urea nitrogen, PTH, phosphorus, and calcium in pcy mice compared with CD1 control mice (Table 1). R-568 treatment, compared with no R-568, lowered serum PTH and blood urea nitrogen in pcy mice but had no significant effect in CD1 mice. However, there was no difference in serum calcium and phosphorus concentration in R-568-treated animals (CD1 or pcy mice) compared with non-treated animals.

The effect of R-568 on the kidneys is shown in Table 1 and Figure 1. The pcy mice had significant renal pathology, with enlarged kidneys, increased weight of kidneys, and fibrocystic histopathology. R-568 treatment prevented much of the renal histopathology in the early stage in NPHP in the pcy mouse. For example, R-568 significantly decreased the total kidney weight (0.66 ± 0.21 versus 0.93 ± 0.21 g, $P < 0.05$) in pcy mice but had no effect in CD1 mice. R-568 also reduced the KW%BW in pcy mice (3.3 ± 0.8 versus 4.8 ± 1.1, Table 1, $P < 0.05$). There was also a significant reduction in cystic change and fibrosis with R-568 treatment. R-568 significantly decreased cyst volume density (%) in pcy mice (Table 1 and Figure 1, $P < 0.05$) and cyst volume (0.20 ± 0.11 with R-568 versus 0.38 ± 0.16 ml without R-568, $P < 0.05$, Table 1). Furthermore, R-568 treatment reduced renal fibrosis (Table 1). These results demonstrate the efficacy of R-568 treatment in slowing early-stage renal disease in this animal model of NPHP.

The effect of R-568 on late-stage NPHP in pcy mice

As shown in Table 2, all three treatment groups (R-568, Ca^{2+}, and R-568 + Ca^{2+}) reduced serum PTH levels compared with control ($P < 0.05$), with no difference between the three treatment groups. Calcium (with or without R-568) significantly decreased serum phosphorus and increased calcium levels versus control and R-568 alone. In contrast, R-568 alone decreased serum calcium (Table 2). None of the treatments significantly reduced blood urea nitrogen levels, but there was a trend in reduction in the R-568-treated group.

Among the three treatment groups, only R-568 alone reduced total kidney weight (R-568: 0.75 ± 0.15 versus control: 0.94 ± 0.30 g, $P < 0.05$; Table 2 and Figure 2), whereas the addition of calcium negated the effects of R-568.

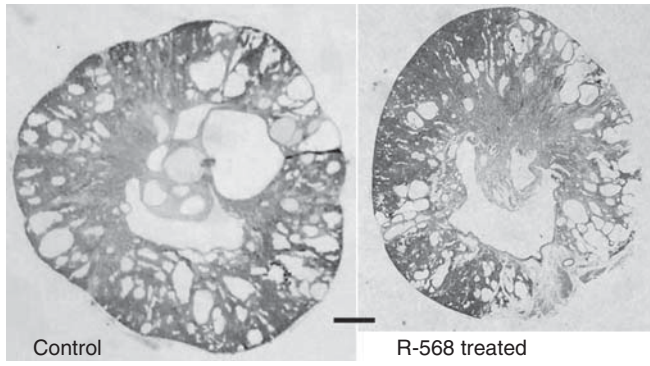

Figure 1 | Renal histopathology in pcy mice treated with R-568 from 5 to 15 weeks of age. At 15 weeks of age, pcy kidney demonstrates diffuse cystic change and near-maximal cystic enlargement (left panel). Treatment with 0.05% R-568 causes a significant delay in the development of cystic pathology (right panel). Scale bar = 1 mm.

Table 1 | Effect of R-568 on the early-stage renal disease in pcy and normal CD1 mice

	pcy		CD1	
Treatment group (4–15 weeks)	Control (*n*=20)	R-568 (*n*=20)	Control (*n*=22)	R-568 (*n*=24)
BUN (mg/dl)	29.9 ± 10.5*	24.8 ± 3.0*#	20.7 ± 3.2	19.7 ± 2.7
PTH* (pg/ml)	201 ± 136*	88 ± 62*#	48 ± 23	26 ± 11
Phosphate (mg/dl)	9.9 ± 2.1*	10.7 ± 2.2*	6.4 ± 1.3	7.0 ± 1.4
Calcium (mg/dl)	8.8 ± 1.2*	8.2 ± 1.3*	5.9 ± 0.7	6.7 ± 1.2#
BW (g)	19.8 ± 2.4*	19.9 ± 1.9*	40.0 ± 8.9	41.8 ± 7.2
KW (g)	0.93 ± 0.21*	0.66 ± 0.21#	0.65 ± 0.15	0.58 ± 0.16
KW%BW	4.8 ± 1.1*	3.3 ± 0.8*#	1.6 ± 0.2	1.4 ± 0.2
Cyst vol density (%)	40.0 ± 9.1	27.7 ± 10#		
Cyst vol (ml)	0.40 ± 0.16	0.21 ± 0.12#		
Fibrosis score	4.0 ± 0.0	3.0 ± 0.3#		

Abbreviations: BUN, blood urea nitrogen; BW, body weight; KW, kidney weight; PTH, parathyroid hormone.
KW%BW is KW as a percent of the total BW; cyst vol density is the cyst volume (assuming 1 g/cc of cyst) expressed as a percent of the total BW; cyst vol is the cyst volume in cubic centimeters (cc) determined from the cyst volume density × KW; and fibrosis score is based on a qualitative, 1+ to 4+ scale.
Data presented as mean ± s.d.
*$P < 0.05$, pcy versus CD1 mice, with or without R-568; #$P < 0.05$, R-568 versus control, pcy, or CD1 mice.

Table 2 | Effect of R-568 on the late-stage renal disease in pcy mice

Treatment group (20–35 weeks)	Control (n=22)	R-568 (n=18)	R-568+Ca^{2+} (n=19)	Ca^{2+} (n=14)
BUN (mg/dl)	60.8 ± 24.3	55.8 ± 19.2	57.3 ± 16.7	71.2 ± 30.4
PTH (pg/ml)	320 ± 235	94 ± 97*	56 ± 25*	75 ± 48*
Phosphate (mg/dl)	8.6 ± 1.9	8.7 ± 1.5	6.8 ± 2.0*#	5.9 ± 2.4*#
Calcium (mg/dl)	8.6 ± 0.3	7.4 ± 0.3*	8.7 ± 0.4#	9.7 ± 0.4*#$
BW (g)	18.1 ± 3.0	17.8 ± 2.0	18.2 ± 3.0	17.7 ± 3.1
KW (g)	0.94 ± 0.30	0.75 ± 0.15*	1.01 ± 0.34#	1.05 ± 0.34#
KW%BW	5.1 ± 1.0	4.2 ± 0.7*	5.5 ± 1.4#	5.9 ± 1.1*#
Cyst vol density (%)	32.2 ± 7.3	28.9 ± 7.5	32.4 ± 7.2	35.8 ± 5.9
Cyst vol (ml)	0.31 ± 0.15	0.26 ± 0.17	0.32 ± 0.14	0.39 ± 0.15
Fibrosis score	4.7 ± 0.5	4.3 ± 0.4	4.1 ± 0.4*	4.9 ± 0.1#

Abbreviations: BUN, blood urea nitrogen; BW, body weight; KW, kidney weight; PTH, parathyroid hormone.
KW%BW is KW as a percent of the total BW; cyst vol density is the cyst volume (assuming 1 g/cc of cyst) expressed as a percent of the total BW; cyst vol is the cyst volume in cubic centimeters (cc) determined from the cyst volume density × KW; fibrosis score is based on a qualitative, 1+ to 4+ scale.
Data presented as mean ± s.d.
*$P<0.05$ compared with control; #$P<0.05$ compared with R-568; and $P<0.05$ compared with R-568+Ca^{2+}.

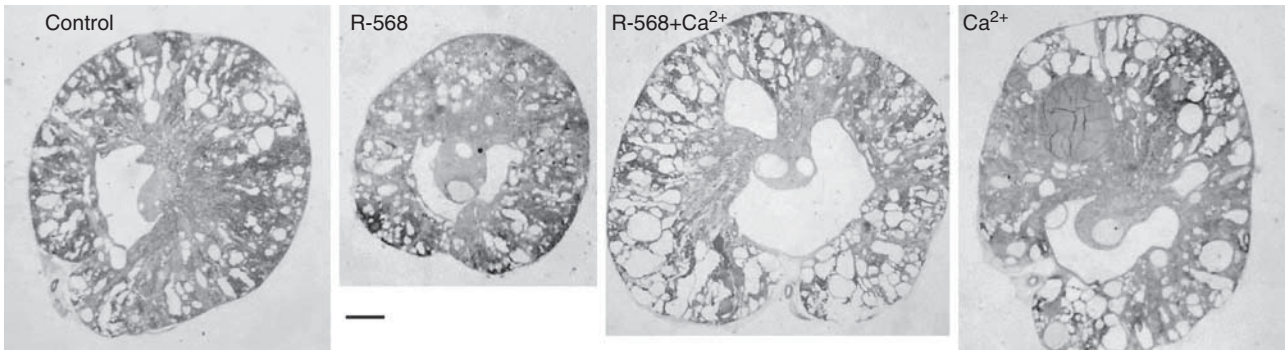

Figure 2 | Renal histopathology in pcy mice treated with R-568 or calcium gluconate from 20 to 35 weeks. Pcy mice were treated with 0.05% R-568 with or without 2% calcium from 20 to 35 weeks of age. The renal cystic changes at 35 weeks are also associated with increased fibrosis in control untreated animals (left panel). The treatment with R-568 alone was associated with a significant reduction in kidney weight and a slight, but not significant, decrease in cystic pathology. However, treatment with Ca^{2+} gluconate (with or without R-568) had no effect on pathology compared to the R-568 alone. Scale bar = 1 mm.

Although there was a trend toward decreased cyst volume density and cyst volume (Table 2 and Figure 2) in R-568-treated animals, this change did not reach significance. Both R-568- and R-568 + Ca^{2+}-treated animals demonstrated a reduction in fibrosis, but only the R-568 + Ca^{2+} reached significance (Table 2). These results demonstrate that R-568 treatment slowed the renal enlargement in the late stage of NPHP.

Expression of CaSR in epithelial lining of the renal cyst in pcy animals

The CaSR is described in the literature as being present in proximal tubules, distal tubules, and the collecting ducts.[11] However, its expression in cyst epithelium is unknown. To confirm that the CaSR is expressed in cystic epithelium, we performed *in situ* hybridization in kidney sections from 35-week pcy mice. The results demonstrate that the CaSR was expressed in the medullary proximal and distal tubules, as expected, and in the epithelial cells lining the renal cysts (Figure 3). This suggests that the observed effect of R-568 on renal cystic disease may be, at least in part, related to direct activation of CaSR in the cysts.

The effect of R-568 on cAMP and proliferation

To determine the mechanism of the beneficial effects of R-568 on cystic kidney disease, we examined three potential mechanisms—reduction in cAMP, increase in intracellular calcium, and reduction in cell proliferation. Treatment of R-568, Ca^{2+}, or R-568 + Ca^{2+} all significantly decreased total kidney cAMP in pcy mice (Figure 4). These treatments also decreased cAMP content in kidney when normalized by kidney weight but only Ca^{2+} alone (57.4 ± 6.8 versus 100.5 ± 16.3 pmol/mg tissue, $P<0.05$) or R-568 + Ca^{2+} (69.9 ± 6.5 versus 100.5 ± 16.3 pmol/mg tissue, $P<0.05$) reached statistical difference. R-568 also decreased cAMP (84.3 ± 7.9 versus 100.5 ± 16.3 pmol/mg tissue, $P=0.2$), but this was not statistically different from control. As all three of these treatments also lowered PTH, we then examined the direct effects of R-568 *in vitro*. As there is no NPHP renal epithelial cell line available, we used human renal epithelial cells isolated from a cystic (ARPKD) kidney and immortalized as an *in vitro* cell model for childhood cystic kidney disease. Our results demonstrated that R-568 lowered cAMP secretion (Figure 5a) and cellular cAMP (Figure 5b), whereas PTH increased cAMP, as has been previously reported in

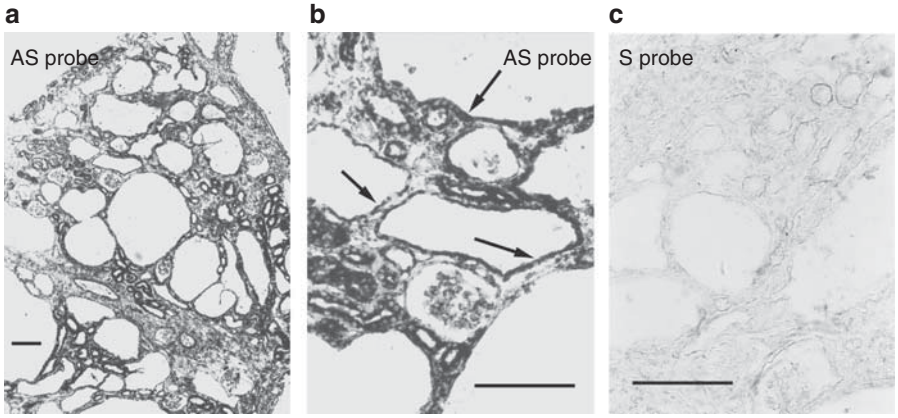

Figure 3 | Expression of CaSR in epithelial lining of the renal cyst in pcy mice. To confirm the localization of the CaSR in cyst epithelial cells, *in situ* hybridization was performed on the paraffin sections of the kidneys from 35-week pcy mice. The results demonstrate that the CaSR was in the epithelial cells lining the renal cysts in pcy mice (**a**, **b**; arrow indicates CaSR expression). For negative controls, serial sections were processed using sense probe (**c**). This suggests that the observed effect of R-568 on renal cystic disease may be directly related to activation of CaSR in the cysts. Scale bar = 1 mm (**a**) and 100 μm (**b**, **c**). AS, antisense; S, sense.

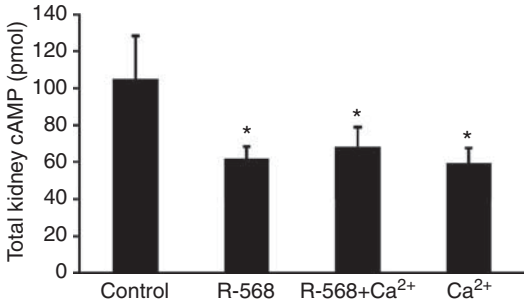

Figure 4 | The effect of R-568 on kidney cyclic adenosine monophosphate (cAMP) content in pcy mice. Pcy mice were treated with or without 0.05% R-568, 2% calcium gluconate (Ca^{2+}), or R-568 with Ca^{2+} from 20 to 35 weeks. The cAMP content was determined and expressed as total kidney cAMP content as well as normalized by kidney weight. The results demonstrated that both R-568 and calcium or the combination of R-568 and calcium significantly decreased total cAMP content in pcy mice. $^*P < 0.05$, all treatments versus control.

cultured bone and kidney epithelial cells.[12,13] Thus, R-568, or lowering PTH, had similar effects *in vivo* and *in vitro*, and cannot fully explain the effects of R-568. We also examined the effect of R-568 on intracellular calcium levels and found R-568 increased intracellular calcium in immortalized human ARPKD renal epithelial cells (Figure 5c). Given the changes in cyst volume density that we observed, we next examined cell proliferation *in vitro* to determine the direct effects of R-568 on cystic epithelial cells. The results demonstrated that R-568 dose dependently decreased cellular proliferation of immortalized ARPKD renal epithelial cells, whereas calcium had no effect (Figure 6). These results suggest that the protective effects of R-568 may be due to multiple mechanisms: reduction of cAMP, decreased proliferation, and increased intracellular calcium, with the latter two mechanisms differentiating R-568 from other PTH-lowering therapies.

DISCUSSION

In the present study, we evaluated an animal model of cystic kidney disease, the pcy model of human NPHP3, and demonstrated that the calcimimetic R-568 was an effective therapy. R-568 treatment significantly decreased kidney weight, cyst volume, cyst volume density, and fibrosis when given from 4 to 15 weeks, and thus prevented the disease progression. This effect was further evident by the reduction in blood urea nitrogen in the treated animals. In later stages of disease in the pcy mouse, R-568 treatment significantly decreased kidney weight, with a trend toward reduction in cyst growth and fibrosis at 35 weeks. We also observed a significant decrease in serum calcium in the late-stage-treated mice. The low calcium may have confounded effects on cyst growth, as the phosphodiesterase responsible for metabolizing vasopressin 2 receptor-generated cAMP is calcium sensitive.[4] Thus, it is possible that the failure of R-568 to reduce cyst volume and fibrosis at later-stage pcy mice, despite the statistically reduced kidney enlargement, may be due to hypocalcemia offsetting the beneficial effects on intracellular calcium and cAMP. However, it is more likely due to differences in the pathogenesis of cyst growth at later stages compared with earlier stages.

We then explored the mechanisms involved in the R-568 amelioration of cystic kidney disease. R-568 decreased total kidney cAMP in pcy mice and inhibited cAMP, decreased cellular proliferation, and increased intracellular calcium in immortalized human ARPKD renal epithelial cells, suggesting the beneficial effect of R-568 on kidney is via both a reduction in cAMP and proliferation and an increase in intracellular calcium, with the latter two unique to R-568 compared with calcium alone. Our studies also support that

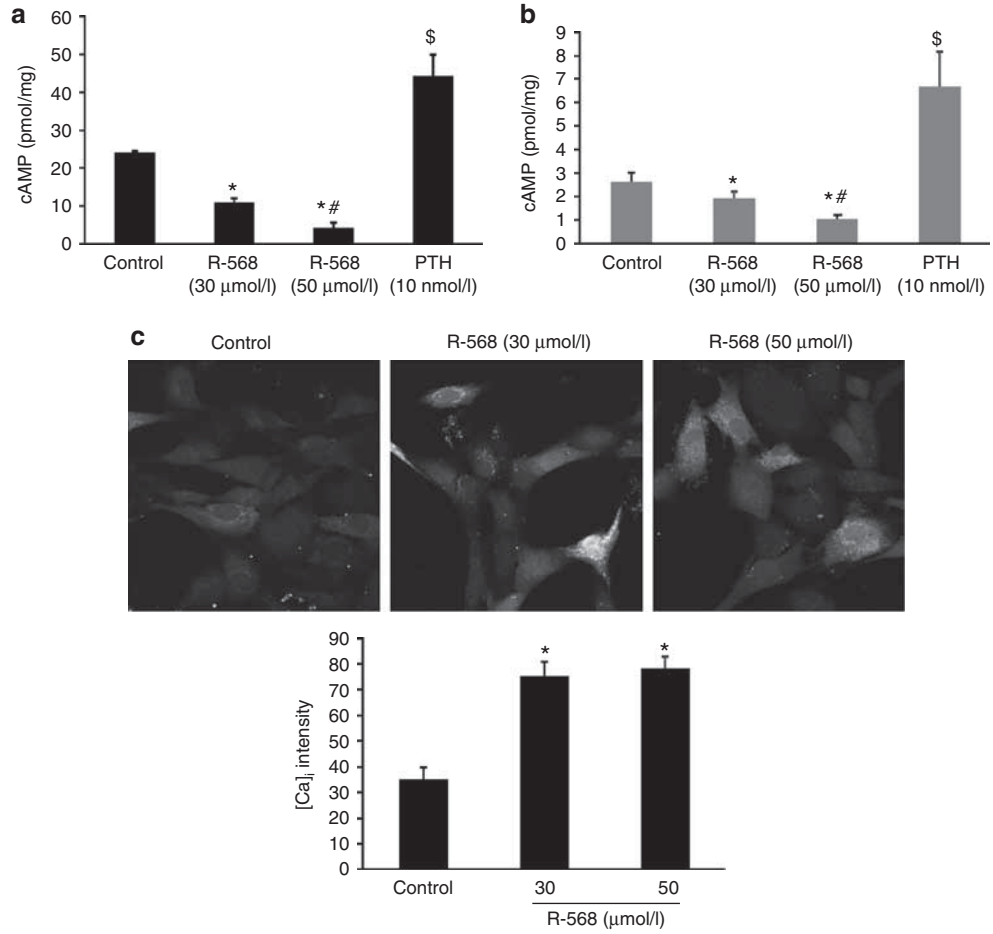

Figure 5 | The effect of R-568 on cyclic adenosine monophosphate (cAMP) content and intracellular calcium in immortalized human autosomal recessive polycystic kidney disease (ARPKD) renal epithelial cells. Human ARPKD-immortalized renal epithelial cells were treated with vehicle (control), R-568 (30 and 50 μmol/l), or parathyroid hormone (PTH; 10 nmol/l) for 24 h. cAMP in conditioned media and cell lysate was determined using an immunoassay kit and normalized by total cellular protein. The results (**a**) demonstrated that R-568 reduced cAMP secretion in a dose-dependent manner, whereas PTH increased cAMP secretion in human ARPKD-immortalized renal epithelial cells. Similarly, R-568 also inhibited cellular cAMP production, and PTH increased cellular cAMP in renal epithelial cells (**b**). Human ARPKD-immortalized renal epithelial cells were treated with or without R-568 for 1 h and intracellular calcium examined using fura 4-AM by confocal microscopy and quantified using Metamorph software. The results demonstrated that R-568 increased intracellular calcium in renal epithelial cells (**c**). $^{*}P<0.05$, versus control; $^{\#}P<0.05$, R-568 (50 μmol/l) versus R-568 (30 μmol/l); and $^{\$}P<0.05$, PTH versus other groups.

the mechanism by which R-568 reduces cAMP is through a direct effect, in addition to a likely indirect effect by decreasing PTH. Our *in situ* hybridization studies confirm that the CaSR is located on cyst epithelium, providing support of a direct role of R-568. Thus, R-568 may be able to 'bypass' the cilia abnormalities to correct defective downstream cell-signaling changes. These studies indicate that R-568 is a potentially useful therapy for inhibition of renal cyst growth, progression, and fibrosis in this animal model of NPHP3 and complement our findings in the Cy/+ model of cystic kidney disease where R-568 prevented late-stage cyst growth and fibrosis.[10]

The finding that R-568 reduces cyst development, growth, and/or fibrosis in both the pcy and Cy/+ animal models suggests a commonality across the diseases. When activated by increases in extracellular ionized calcium, or by the allosteric activator R-568, the CaSR acts through the G_q protein to activate phospholipase C-protein kinase C signaling. This, in turn, leads to increased intracellular calcium and, through the G_i protein, inhibition of cAMP generation.[14] This would reverse abnormalities that are thought important in the pathogenesis of cyst formation.[15,16] Our current study demonstrated that R-568 decreased total kidney cAMP content in pcy mice, suggesting that the beneficial effect on kidney of R-568 may be associated with a reduction in cAMP. Also supporting this mechanism is that lowering cAMP by the therapeutic use of vasopressin 2 receptor antagonists inhibits the development and progression of the renal cystic disease in animal models orthologous to human autosomal dominant polycystic kidney disease ($Pkd2^{-/WS25}$ mice), ARPKD (PCK rats), and NPHP (pcy mice).[17,18] In contrast, use of calcium-channel blockers, which decreases intracellular calcium, accelerates PKD progression.[19] Nephrocystin 3, the protein defect resulting from mutations in the *NPHP3* gene, is localized to primary cilia.[3] The renal cilia are required for the physical and

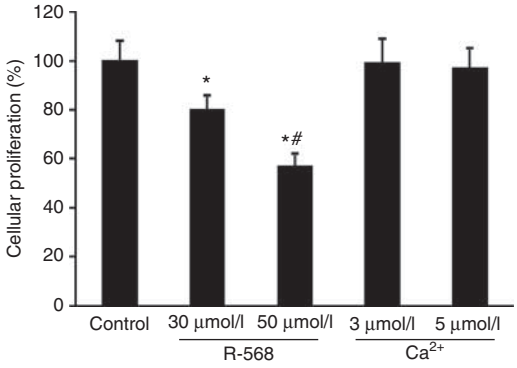

Figure 6 | The effect of R-568 on cellular proliferation in renal epithelial cells. Immortalized human autosomal recessive polycystic kidney disease (ARPKD) renal epithelial cells were treated with vehicle (control), R-568 (30 and 50 μmol/l), or calcium (3 and 5 mmol/l) for 24 h and cellular proliferation determined. The results demonstrated that R-568 dose dependently inhibited cellular proliferation, whereas calcium treatment had no effect in human ARPKD renal epithelial cells. $^{*}P<0.05$, versus control and $^{\#}P<0.05$, R-568 (50 μmol/l) versus R-568 (30 μmol/l).

chemical stimulation of intracellular calcium.[20,21] and thus, when genetically altered, lead to decreased intracellular calcium and increased cAMP. However, the differences that we observed between R-568 and calcium treatment, despite similar effects on cAMP, indicate that changes in cAMP cannot completely explain the beneficial effects of R-568.

Other abnormalities common to cystic diseases are defects in planar cell polarity, increased rates of proliferation and apoptosis, expression of a secretory phenotype, and remodeling of the extracellular matrix.[4] It is not clear whether these abnormalities are directly related to changes in intracellular calcium and cAMP or occur through other signaling pathways. Our finding that R-568 directly reduced proliferation *in vitro* suggests an alternative or additional mechanism by which R-568 may reduce cyst growth. Activation of the CaSR is also known to reduce cell proliferation in parathyroid cells,[22] keratinocytes,[23] colonic epithelium,[24] and cancer cells.[25,26] In addition, activation of the CaSR also appears to affect terminal differentiation in these cells and perhaps can explain the reduced fibrosis we observed. The cell signaling pathways involved are diverse in these cell types. However, Wnt signaling is a possibility, as the gene defect in NHPH type II, inversion, is known to lead to alterations in Wnt signaling.[27] Another study demonstrated that mice lacking the jouberin protein develop NHPH due to a decrease in Wnt signaling and nuclear β-catenin accumulation.[28] Clearly, more research is required to fully elucidate the role of this signaling pathway in cystic epithelium.

It is important to note that Wang *et al.*[29] did not find an effect of R-568 on the PCK rat and PKD2$^{-/ws25}$ mouse using 0.05 and 0.1% R-568 mixed in food (~25 or 50 mg/kg). They treated PCK rats from 3 to 10 weeks and PKD2$^{-/ws25}$ mice from 3 to 16 weeks. In these studies, they did not find a reduction in cyst volume or kidney size in either model. However, they did find a reduction in fibrosis in R-568-treated PCK rats. This implies that different cell signaling may be involved in fibrosis and cyst growth. In some series, cyst growth in the PCK rat is greater at later stages of disease,[30] and thus it is also possible that the time points examined may have been too early to observe an alteration in cyst growth. More studies are required to understand the differences observed in different animal models of cystic disease.[29]

In conclusion, treatment of pcy mice, an orthologous animal model of the cystic disease NPHP, with R-568 inhibited progression of cyst growth and renal fibrosis. R-568 was less effective in later-stage NPHP but did significantly reduce kidney weight. Taken together, these results offer hope that calcimimetics may be a potential new therapy for these devastating childhood diseases, although the risk and benefits will need to be determined in a clinical trial to ensure that hypocalcemia is not treatment limiting.

MATERIALS AND METHODS

Animal models and study design

The *pcy* mouse is a model of NPHP that is caused by a missense mutation in *NPHP3*, the gene mutated in adolescent NPHP.[31] The pcy mouse develops renal cysts beginning at 5 weeks and then plateaus between 15 and 20 weeks of age. Thereafter, the cysts enlarge, culminating in severe azotemia at 35–40 weeks.[17] Therefore, we evaluated two different treatment periods: a preventive stage where animals were treated from 4 to 15 weeks and a treatment phase where animals were treated from 20 to 35 weeks. For the prevention study, male and female pcy or wild-type CD1 mice were provided with the following treatment diets: (1) control diet: LabDiet no. 5002, diet without any additive (contains 0.862% Ca^{2+} and 0.62% P); (2) R568 diet: LabDiet (TestDiet, Richmond, IN) no. 5002, diet with 0.05% R-568 (to deliver a dosage of ~50 mg/kg/day). For the later-stage pcy mouse study, where there is established secondary hyperparathyroidism, two additional treatment arms were added to differentiate direct effects of R-568 from that of calcium, which also lower PTH: (3) R-568 + Ca^{2+}: Pcy mice fed the same diet as group 2, with the addition of 2% calcium gluconate to the drinking water (R-568 + Ca^{2+}) and (4) Ca^{2+}: Pcy mice fed same diet as group 1, with the addition of 2% calcium gluconate to the drinking water (Ca^{2+}). We only utilized these latter two treatments in the older animals, as we anticipated much greater reductions in PTH at this stage of CKD.

The pcy mice were provided food and water, and maintained on a 12 h/12 h, dark/light cycle. At termination, rodents were weighed, anesthetized with sodium pentobarbital 100 mg/kg given intraperitoneally, and blood collected via an intracardiac extraction. A laparotomy was performed and the kidneys flushed with saline. The left kidney was weighed and frozen, whereas the right kidney was weighed and transverse sections fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer for processing for paraffin embedment. Studies were approved by the Indiana University Institutional Animal Care and Use Committee.

Plasma biochemistries

Sera were assayed for urea nitrogen (Sigma Urea Assay kit no. 640, Sigma-Aldrich, St Louis, MO), intact PTH (ELISA kit from ALPCO Diagnostics, Salem, NH), and calcium and phosphate concentrations (Pointe Scientific, Canton, MI).

Histomorphometric analysis

Transverse sections of the kidney, including cortex, medulla, and papilla, were paraffin processed and sections stained with hematoxylin and eosin to measure cyst volume or with picrosirius red stain to assess fibrosis.[30] In a blinded analysis, random regions of the cortex/outer medulla were photographed and evaluated for cystic change (cyst volume density, Vv). The amount of cystic change was determined using point count stereology methods, as previously described.[10,17] Briefly, the percentages of intersects from the grid that overlie cysts were determined as a percentage of the total grid intersects that overlie the kidney section, so that cyst Vv was expressed as a percentage of the kidney section, which was extrapolated to the percentage of the kidney mass. The total cystic volume was determined by multiplying the Vv by the kidney weight to determine the cubic centimeters (ml) of total cyst volume. The fibrosis score was determined from picrosirius red stain-stained sections and the percentage of fibrosis evaluated within the interstitial/intertubular space using a semiquantitative 1+ to 4+ score: 1+ indicated a minimal amount of fibrosis (<5% if the intertubular region) and 4+ indicated >60%. This method was chosen, as the fibrosis was limited to the interstitial, intertubular regions, and an automated, regional assessment of staining would be compromised by the amount of cystic pathology, as cysts do not have fibrosis within their lumina.

In situ hybridization

To confirm localization of the CaSR in cyst epithelial cells, *in situ* hybridization was performed on the paraffin sections of the kidneys from pcy mice, as previously described.[32] The specimens were prehybridized for 2 h at 42°C and hybridized with the labeled riboprobe (5 ng/μl) for 16 h. Riboprobes were made by *in vitro* transcription of linearized plasmids (PGEM-T) containing cDNA for mouse or rat CaSR (kindly provided by Drs Bill Richard and Charles Henley from Amgen Thousand Oaks, CA). *In vitro* transcription was performed in the presence of digoxigenin–deoxyuridine 5′-triphosphate and T7 RNA polymerase (antisense probe) or Sp6 RNA polymerase (sense probe) according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN). Specimens were washed under stringent conditions. After hybridization, sections were washed for 30 min in 2 × SSC at room temperature and then 1 h each in 1 × SSC, 0.5 × SSC, and 0.1 × SSC in the presence of 50% formamide at 48°C. Probe binding was localized by a colorimetric reaction with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics). Methyl-green was used for counterstaining. For negative controls, serial sections were processed using sense probe.

Content of cAMP in whole kidneys from pcy mice and immortalized human ARPKD renal epithelial cells

Kidneys were ground to fine powder under liquid nitrogen in a stainless steel mortar and homogenized in 10 volumes of cold 5% trichloroacetic acid in a glass–teflon tissue grinder. After centrifugation at 600 *g* for 10 min, supernatants were extracted with three volumes of water-saturated ether. After drying the aqueous extracts, reconstituted samples were processed without acetylation using an enzyme immunoassay kit (Assay Designs, Plymouth Meeting, PA).

Human-immortalized renal epithelial cells isolated from ARPKD patients[33] were treated with or without R-568 for 24 h. Conditioned media were collected and cellular lysate isolated. cAMP was determined using an immunoassay kit (Assay Designs) and normalized by total cellular protein content.

Intracellular Ca^{2+} measurement

Isolated human-immortalized renal epithelial cells were treated with or without R-568 for 1 h. Cells were then loaded with 5 μmol/l fura 4-AM (Molecular Probes, Eugene, OR), a fluro calcium indicator, in Hank's balanced saline solution for 30 min at 37°C. Cells were rinsed and incubated for an additional 30 min with Hank's balanced saline solution alone to allow for complete de-esterification of the fluorescent probe. Intracellular calcium changes were examined by confocal microscopy and quantified using Metamorph software (Molecular Device, Sunnyvale, CA), as previously published.[34]

Cellular proliferation assay

Human-immortalized renal epithelial cells isolated from ARPKD patients[33] were seeded in a 96-well culture plate and treated with various concentrations of R-568 or $CaCl_2$ for 24 h. Cellular proliferation was determined by the Promega Cell Titer 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacture's instructions.

Statistical analysis

Comparisons between groups were made using one-way or two-way analysis of variance, with least significant difference comparisons of the means or Student's *t*-test as appropriate. Data are expressed as mean ± s.d. Analyses were done using StatView software (SAS, Cary, NC).

DISCLOSURE

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